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(54) A reporter gene based process for the screening of anti-tuberculosis drugs by using essential and regulatory genes of mycobacteria as drug target

(57) The present invention relates to an *in vivo* drug screening method taking advantage of the yeast two-hybrid system provides a method of using the *whiB* genes which are regulatory genes of mycobacteria, whose functions have not been reported so far, as drug target by using the yeast *Saccharomyces cerevisiae* as a surrogate host.

EP 1 245 683 A1

Description

FIELD OF THE INVENTION

[0001] This invention relates to the development of a reporter gene based drug screening system against tuberculosis by using essential regulatory genes of *Mycobacterium tuberculosis* H37Rv as target. Such regulatory genes are more particularly the *whiB* genes of mycobacteria whose functions are essential for the survival and normal growth of mycobacteria.

BACKGROUND OF THE INVENTION

[0002] The Streptomycetes are dimorphic organisms. After reaching the late log phase of growth, the substrate mycelia differentiate into the aerial mycelia. The tip of the aerial mycelium then differentiates into a chain of spores. Each spore represents a single cell and is separated by a septum. In 1992, Davis and Chater (Davis, N.K. and Chater, K.F. 1992. Mol. Gen. Genet. 232: 352-358) reported that any mutation in the *whiB* gene of *Streptomyces coelicolor* A3 (2) results into a non sporulating organism. These mutants were also white in colour since they had lost capability to produce deep redish blue pigment which is a characteristic of the wild type strain of *Streptomyces coelicolor* A3(2). It was further confirmed that a fully functional *whiB* gene is essential for the sporulation of the *Streptomyces coelicolor* A3(2) and *whiB* gene may be a transcription activator. A *whiB* homologue was also reported from *Streptoverticillium sps, Streptomyces aurofaciens* and *Rhodococcus opacus* (Kormanec and Homerova, 1993 Nucl. Acid Res. 21:2512; Seibert,V., Kourbatova, E.M., Golovvela, L.M. and M. Schlomann. 1998. 180: 3503-3508; Soliveri, J.E., Vijgenboom, E., Granozzi, C., Plaskitt, K.A. and K.F. Chater. 1993. J.Gen.Microbiol. 139:2569-2578). However, unexpectedly, the genome sequence of *Mycobacterium tuberculosis* H37Rv showed the presence of four genes that is *whiB1*/ Rv3219-254bp, *whiB2*/Rv3260c-269bp, *whiB3*/Rv 3416-308bp and *whiB4*/Rv3681c-302bp whose deduced amino acid products were similar to the *whiB* gene of *Streptomyces coelicolor* A3(2). The amino acid sequences of *whiB* genes of *M. tuberculosis* H37Rv show 32-35 per cent homology to the amino acid sequences of *Streptomyces whiB* genes. Although the homology is relatively low, the general property of the predicted protein remains conserved. General morphology of mycobacteria is bacillus unlike the species of streptomycetes, which are filamentous. So far, sporulation of mycobacteria has not been reported. Therefore the presence of *whiB* like genes, which controls the sporulation, in a non-sporulating organism is highly intriguing. The predicted amino acid sequence of *whiB* gene suggests that the *whiB* gene may code for a transcription activator. If that were so then *whiB* genes would be a regulatory gene. However so far it has not been reported that the *whiB* genes indeed code for a transcription activator. If the *whiB* genes are indeed a set of regulatory genes then the question is what kind of genes it controls? Recently, Gomez and Bishai (Gomez, J.E. and Bishai, W.R., 2000. Proc. Natl. Acad. Sci. 97: 8554-8559) have shown that a *whiB2* homologue of *Mycobacterium tuberculosis* H37Rv is present in *Mycobacterium smegmatis* and is essential for its survival. *Mycobacterium smegmatis* is a fast growing and non-pathogenic organism. However no report or patent could be found related to the invention described in this invention.

[0003] The present invention describes that out of the four *whiB* genes originally described in the *Mycobacterium tuberculosis* H37Rv genome sequence, the *whiB1* /Rv 3219 is essential to the survival of the *Mycobacterium bovis* BCG and *whiB3* /Rv 3416 appears to control septa formation during cell division. The properties of these genes were not reported in the genome sequence of *Mycobacterium tuberculosis* H37Rv nor it has been published in the literature (Cole et al. Nature 1998. 393: 537-544.).

[0004] Tuberculosis is still one of the major killers of human lives in India and in most of the developing countries. The spread of HIV has compounded the problems of tuberculosis because several species of mycobacteria, which were otherwise not known to infect humans, have also been found to be associated with the HIV infected patients. More than often these new pathogens are not sensitive to the conventional therapy of tuberculosis. Further the incidence of the tuberculosis caused by the drug resistant mycobacteria are also increasing with alarming rate. These resistant bacteria do not respond to the conventional therapy being in practice. Thus there is need to find either new drugs or first to find new drug targets that have not yet developed capability to modify themselves and then search for a drug, which would attack at a particular target. Search for a new drug without any specific target usually lead to the rediscovery of known drugs. Secondly, the mode of action of the new drug discovered by random search is usually not known, thus the study of pharmaco-kinetics can be a laborious process. Further, if the organism develops resistance to the new drug as well then modification of these drugs which, would suit to the target of resistant organism would be very difficult. With the advancement in computer simulation studies and modeling software, it is possible to design a drug if the target is known.

[0005] The present invention uses the DNA binding property of the *whiB* genes of *Mycobacterium tuberculosis* H37Rv that has been demonstrated for the first time in this invention. The invention is based on the activation of the reporter gene *lacZ,* which produces the enzyme β-galactosidase. In a normal circumstance the β-galactosidase activity remain

repressed because the *whiB* genes, which code for a DNA binding protein, can bind to the *lexA* operators present in certain strains of *Saccharomyces cerevisiae* as well as in reporter plasmids. Binding of *whiB* gene products with the operator's sequence results in the repression of the transcription of the *lacZ* gene, which produces β-galactosidase enzyme. However if a drug binds to the *whiB* genes then its products will not be available for binding to the operators and the *lacZ* transcription will continue. Activation or repression of β-galactosidase can be monitored either by adding 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) into the growth medium, which produces blue color or by adding o-nitrophenol β-D-galactopyranoside (ONPG) which produces yellow color in presence of β-galactosidase. Intensity of the color production is directly related to the activation of β-galactosidase enzyme and therefore would be directly related to the binding capability of a drug. In other words, a strong binder to the target will allow strong activation of β-galactosidase, however, a poor binder to the target would permit low activation of β-galactosidase enzyme. The method in this invention is fully compatible to any automated High-Through-Put screening system or any other automated or non-automated screening system.

[0006] The present invention is thus based upon the need to find out a new drug target in *Mycobacterium tuberculosis* H37Rv and develop a drug screening system based upon the identified target. To suit to the fast developing technology and urgent need to find a better cure, it is desired that the screening system is compatible to any automated High-Through-Put screening or any other mechanised screening procedure.

OBJECTS OF THE INVENTION

[0007] The main objective of the present invention is to provide a new drug target, which would facilitate target specific screening of anti-tuberculosis drugs.

[0008] Another objective of the present invention is to develop *in vivo* drug screening system, which is fast and uses the properties of the target gene.

SUMMARY OF THE INVENTION

[0009] The present invention relates to an *in vivo* drug screening method taking advantage of the yeast two-hybrid system, known in the literature. The invention also describes that the *whiB* genes are essential regulatory genes of *Mycobacterium tuberculosis* H37Rv and appear to be conserved amongst the pathogenic and slow growing mycobacteria. Thus the novelty of the method of the invention is to use the *whiB* genes which are regulatory genes of mycobacteria, whose functions have not been reported so far, as drug target by using the yeast *Saccharomyces cerevisiae* as a surrogate host.

DETAILED DESCRIPTION OF THE INVENTION

[0010] To accomplish the aforesaid and other objectives, it is essential to demonstrate that a functional target gene is essential for the survival of the *Mycobacterium tuberculosis* H37Rv. The sporulation gene homologue of *Streptomyces coelicolor* A3(2) was found to be present in *Mycobacterium tuberculosis* H37Rv. Since mycobacteria are not known to sporulate, it was assumed that these sporulation homologues would have yet unknown but important function. It has been assumed that the *whiB* genes may act, as a transcription activator therefore would have regulatory function. It is further assumed that a regulatory gene, which controls sporulation, yet present in a non-sporulating organism, may indeed be an essential gene.

[0011] The *in vivo* drug screening method described in this invention takes advantage of the yeast two-hybrid system, the art known in the literature. The invention also describes that the *whiB* genes are essential regulatory genes of *Mycobacterium tuberculosis* H37Rv and appear to be conserved amongst the pathogenic and slow growing mycobacteria. Thus the novelty of the invention is to use the *whiB* genes which are regulatory genes of mycobacteria, whose function have not been reported so far, as drug target by using the yeast *Saccharomyces cerevisiae* as a surrogate host.

[0012] *Mycobacterium tuberculosis* H37Rv is a slow growing organism and is highly virulent. Thus, the use of live organism poses severe health hazard. Therefore it was essential to develop a screening system in a different host organism, which is non-pathogenic, fast growing and also the method is adaptable to High-Through-Put screening or any other automated screening systems.

[0013] Accordingly, the invention provides a reporter gene based method for the screening of anti-tuberculosis drugs comprising using *whiB* like genes (*whiB*1, *whiB*2 whiB3 *whiB*4) present in *Mycobacterium tuberculosis* H37Rv, *Mycobacterium bovis* BCG and *Mycobactereium leprae* having DNA and protein sequence as shown in sequence ID 1 to 4 as drug target for the screening of anti-tuberculosis and anti-leprosis drugs.

[0014] The *whiB*3 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG can be used for the screening of anti-tuberculosis drugs. The presence of functional *whiB*4 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG is important for their normal growth. The *whiB*4 gene of *Mycobacterium tuberculosis*

H37Rv and *Mycobcaterium bovis* BCG code for a DNA binding protein.The drugs against *whiB*2 and the *whiB*4 genes of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG will be particularly useful where drug resistance has developed against the *whiB*1 and *whiB*3 genes or where the *anti-whiB*1 and *anti-whiB3* drugs are allergic or toxic. The source of *whiB* like genes can be *Mycobacterium avium, Mycobacterium fortuitum, Mycobacterium gastri, Mycobacterium kansasii, Mycobacterium leprae, Mycobaclerium marinum, Mycobacterium microti, Mycobacterium phlei, Mycobacterium scrofulaceum, Mycobaclerium smegmatis* and *Mycobacterium xenopi* and related organisms.

[0015] Further, the present invention provides for a reporter gene based method for the screening of anti-tuberculosis drugs by using essential and regulatory genes of mycobacteria as drug target and comprises the following steps:

(a) amplifying the *whiB* genes of *Mycobacterium tuberculosis* H37Rv by polymerase chain reaction (PCR);
(b) cloning the products obtained in step (a) into plasmid vectors such as pUC19 or pBluescript SK^+ or SK^- and transforming into *E.coli*;
(c) amplifying *whiB* genes of *Mycobacterium bovis* BCG by using the oligonucleotide primers of *Mycobacterium tuberculosis* H37Rv;
(d) cloning the product obtained in step (c) into a plasmid vector pUC19 or pBluescript SK^+ or SK^-; and sequencing the products to confirm the sequence of cloned fragment;
(e) constructing a gene disruption integrative vector by inserting a kanamycin cassette in between the *whiB* gene sequences of *Mycobacterium tuberculosis* H37Rv;
(f) constructing a gene disruption integrative vector by inserting a kanamycin cassette in between the *whiB* gene sequences of *Mycobacterium bovis* BCG;
(g) amplifying *whiB* genes of *Mycobacterium tuberculosis* H37Rv by using oligonucleotide primers carrying either *Eco*RI, or *Bam*HI restriction enzyme sites at the 5' end and *Xho*I restriction enzyme site at the 3' end;
(h) digesting the fragment as obtained in step (g) with suitable restriction enzymes and then cloning the fragment into a *E. coli*/*Saccharomyces cerevisiae* shuttle vector pEG202 to produce the cloned gene as a *LexA* fusion protein and transforming the recombinant plasmid into *E.coli;*
(i) preparing large amount of the desired recombinant plasmid and then transforming the recombinant *whiB*1, *whiB*2,*whiB*3, and *whiB*4/pEG202 into the *Saccharomyces cerevisiae* strains EGY48 and EGY191,
(j) co-transforming the pJK101, pSH 18-34, pSH17-4, pJG4-5 and pRFHM vectors into the *Saccharomyces cerevisiae* strains EGY48 and EGY191 already harboring *whiB* genes in the pEG202 vector and then selecting for the clones, which complements for uracil and histidine + uracil or histidine + uracil + tryptophan auxotrophy;
(k) growing the *Saccharomyces cerevisiae* transformants in the yeast nitrogen base medium containing: galactose / raffinose+, ura-, BU salt+, X-gal+; his-, ura-. BU salt+, X-gal+, glucose; galactose / raffinose+, ura-, his-, leu-; glucose+, ura-, his-, leu-; galactose / raffinose+, ura-, his-, trp-, leu- and glucose+, ura-, his-, trp-. leu-plates, and
(l) using the DNA binding property of the *whiB*1, *whiB*2 and *whiB*3 for the screening of anti-tuberculosis drugs.

[0016] In an embodiment, the restriction enzymes are selected from *Eco*RI, *Xma*I *Bam*HI, *Sal*I, *Nco*I, *Not*I, *Xho*I, *Sal*I and *Pst*I.

[0017] In another embodiment, *whiB*1 gene is essential for the survival of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

[0018] In still another embodiment, the *whiB*2 gene is essential for the normal growth of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

[0019] In yet another embodiment, the *whiB*3 gene is essential for the cell division of a *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

[0020] In an embodiment, the *whiB*4 gene is essential for the normal growth of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

[0021] In another embodiment, the *whiB*4 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG is a transcription activator.

[0022] In yet another embodiment, the *whiB*1, *whiB*2 and *whiB*3 genes of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG code for a DNA binding protein.

[0023] In still another embodiment, the whiB1, whiB2 and whiB3 genes of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG repress the activation of β-galactosidase genes after binding to the Lex A operator or any other like operators.

[0024] In an embodiment, the DNA binding domain of the *whiB* genes is situated within the 15 amino acids at the carboxy- terminus of the protein.

[0025] In another embodiment, the DNA binding property of the *whiB* genes has been used to screen those anti-tuberculosis drugs which attack the *whiB* genes. The method of the invention can be used for any gene, which codes for a DNA binding protein. The method is compatible to any High — Through- Put or automated screening system.

[0026] The details of the invention are:

[0027] The *Mycobacterium tuberculosis* H37Rv genome sequence was obtained from the internet site http://www.sanger.ac.uk and the sequence of the *whiB* genes were recovered. The authors, (Cole et al. Nature, 1998:393, 537-543) have annotated these genes as: *whiB1*/Rv3219 *(255bp), whiB*2/Rv3260c (270bp), *whiB*3/Rv3416 (309bp) and *whiB*4/Rv3681c (303bp). The sequence of oligonucleotide primers designed to amplify the *whiB* genes were as follow:

F 5' acccgttaccagccaagaag 3' and R 5' gggacggttgatgctgtag 3' for *whiB*1
F5' ggccgggtcagatgatc 3' and R 5'accgcatctgagtttgg 3' for *whiB*2
F 5' atgccacagccggagcagctac 3' and R 5' ttaagctgtgcggcggatgcc 3' for *whiB*3
F 5' ctatccggcggtgccggtgcg 3' and R 5' gtggtacgcagcgtagacgcg 3' for *whiB*4

The sequences of the genes are shown as sequences ID 1 to 4 separately.

[0028] The PCR was done by standard procedure. The PCR products were separated on 1 per cent agarose gel and the amplified bands were removed by cutting the gel. The PCR amplified DNA was purified by using a commercially available purification kit. Cloning and then transformation of the PCR fragments were done by standard procedure. The sequence of cloned fragments was confirmed by standard procedure.

[0029] Once the identity of the cloned fragments was confirmed, the *Mycobacterium tuberculosis* H37Rv *whiB* genes were used as probe to check whether similar genes were present in the *Mycobacterium bovis* BCG or not. The *Mycobacterium bovis* BCG chromosomal DNA was prepared by a published method (G.P.S. Raghava, R.J.Solanki, V. Soni and P. Agrawal. Biotechniques. 2000. 29:108-116). A standard Southern hybridization protocol was followed to confirm the presence of *whiB* genes in *Mycobacterium bovis* BCG as well.

[0030] As described for the *Mycobacterium tuberculosis* H37Rv, all the four *whiB* genes in *Mycobacterium bovis* BCG were also PCR amplified. The oligonucleotide primers and PCR conditions were identical in both the cases. The PCR products were separated in an agarose gel, purified, cloned and sequenced as described in case of *Mycobacterium tuberculosis* H37Rv.

[0031] The sequence alignment using commercial computer software confirmed that the *whiB* gene sequences of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG are identical.

[0032] By using commercially available computer software Gene-Runner, restriction enzymes sites within the *whiB* sequences were found. Based on the sequence analysis results following enzyme sites were selected to generate vectors that can be used for gene disruption in *Mycobacterium bovis* BCG by the process of homologous recombination. To generate a vector, which could be used for gene disruption following constructs were created:

(a) for the *whiB*1 kanamycin cassette was inserted at *Pvu*II site;
(b) for *whiB*2 two independent constructs were made at *Mlu*I and at *Hae*III sites;
(c) for *whiB*3 also two constructs were made at *Eco*RI site and at the *Cla*I site;
(d) for *whiB*4 at the *Sac*II site.

[0033] The *whiB* recombinant clones were digested with restriction enzymes. However, whenever the recombinant clone had more than one site for a particular enzyme then the purified *whiB* fragments were digested and then ligated with the kanamycin cassette, which codes for kanamycin resistance. The *E. coli* strain was transformed and clones were selected for ampicillin and kanamycin resistance. Since these clones do not have mycobacterial origin of replication they will not survive within the mycobacteria unless they are integrated in the mycobacteria genome. Electrocompetent *Mycobacterium bovis* BCG cells were prepared using the art known in the literature and transformed with 1μg of purified vector DNA. In each tube 1ml of 7H9 Middlebrook's medium was added and incubated at 37°C for 48hrs. The culture was then plated on a 7H10 Middlebrook's medium containing both ampicillin and kanamycin and incubated at 37°C. After three weeks of growth the colonies were again plated on kanamycin plates and allowed to grow at 37°C. In the kanamycin plates the colonies could be seen only after 6 weeks of incubation which suggested that the *whiB* disruption is deleterious for the growth of *Mycobacterium bovis* BCG. Disruption of individual *whiB* genes had following effects on the growth and survival of *Mycobacterium bovis* BCG:

1. the *whiB*1 disruption is lethal.
2. the *whiB*2 disruption makes the cells very slow growing and the colonies are very small in size.
3. the *whiB*3 disruption makes the cells mycelial which clearly suggests that this disruption is controlling septa formation.
4. the *whiB*4 disruption also had similar effect like *whiB*2 disruption.

[0034] To demonstrate the nature of the *whiB* gene yeast two-hybrid system was used. This art is well known in the literature. In principle the system has been developed in such way that the trans-activation domain and the DNA binding domains are in two different plasmids. Unless both the domain come together protein-protein interaction will not take

place thus a gene will not get activated. However, the system also allows one to check whether the gene in question codes for DNA binding protein or a transcription activator.

[0035] The *whiB*1, *whiB*2 and *whiB*4 genes were PCR amplified using oligonucleotide primers, which had *Eco*RI site at their 5' end and *Xho*I site at their 3' end. The *whiB*3 gene was amplified with the primers having *Bam*HI site at the 5' end and *Xho*I site at the 3' end. After digesting with the appropriate restriction enzymes these genes were cloned into the *E.coli* / *Saccharomyces cerevisiae* shuttle vector pEG202. After selecting for ampicillin resistant colonies, the recombinant clones were selected for his colonies in *Saccharomyces cerevisiae* EGY 48 and EGY 191. The his- clones were then transformed with the plasmids: pJk101, pSH18-34, pJG4-5, and pRFHM either singly or in combinations. The clones which complemented either for uracil, uracil + histidine or uracil + histidine + tryptophan were selected. These clones were then tested for either activation or repression of β-galactosidase activity by growing them in the plates containing yeast-nitrogen based medium and following supplements:

(1) glucose $^{+}$, ura^{-} BU salt^{+} +X-gal (no color)
(2) galactose/raffinose^{+}, ura^{-} + BU salt^{+} + X-gal (no color by all the four *whiB* genes only when pJK101 is present but pJK101 produced bright blue color, suggesting that *whiB* genes are expressed in the *Saccharomyces cerevisiae* and repress the activation of *lacZ* gene.)
(3) glucose^{+}, ura^{-},his^{-},leu^{-} (no growth)
(4) galactose / raffinose^{+}, ura^{-}, his, leu^{-} (no growth of *whiB*1, *whiB*2, and *whiB*3 but some growth was seen *of whiB*4 in presence of pSH18-34)
(5) galactose/ raffinose+, ura-, his-, tryp-, leu- (only *whiB*4 showed some growth after three days of incubation in presence of pSH18-34 and pJG4-5, suggesting that the *whiB*4 gene is a weak transcription activator)
(6) glucose+, ura-, his-, tryp-, leu- (no growth).

[0036] The method of the present invention is illustrated in the examples given below which should not, however, be construed to limit the scope of the present invention.

Example 1

[0037] The *Mycobacterium tuberculosis* H37Rv chromosomal DNA was prepared as following a published method (G.P.S. Raghava, R.J.Solanki, V.Soni and P. Agrawal. Biotechniques. 2000. 29: 108-116).

[0038] All the four *whiB* genes of *Mycobacterium tuberculosis* H37Rv were PCR amplified using a standard protocol. The oligonucleotide primer's and PCR conditions were as follows:

F 5' acccgttaccagccaagaag 3' and R 5' gggacggttgatgctgtag 3' for *whiB*1

F5' ggccgggtcagatgatc 3' and R 5'accgcatctgagtttgg 3' for *whiB*2

F 5' atgccacagccggagcagctac 3' and R 5' ttaagctgtgcggcggatgcc 3' for *whiB*3

F 5' ctatccggcggtgccggtgcg 3' and R 5' gtggtacgcagcgtagacgcg 3' for *whiB*4

[0039] The PCR products were separated on 1 per cent agarose gel and the amplified bands were removed by cutting the gel. The PCR amplified DNA was purified by using a commercially available kit.

[0040] The ends of the purified DNA were phosphorylated using T4 Pol-Kinase and then cloned into the *Sma*I site of the dephosphorylated pUC19 vector. The recombinant plasmids were transformed into *E.coli* strain and selected for the colonies, which did not produce blue color. The white colonies were recovered from the plates and were grown on a fresh medium containing 100ug/ml ampicillin and 25ug/ml of kanamycin as a selection marker. The clones were sequenced using a standard method, well known in the art. Thus this example establishes the cloning and confirmation of the four *whiB* genes of *Mycobacterium tuberculosis* H37Rv.

Example 2

[0041] The *Mycobacterium tuberculosis* H37Rv is a highly virulent strain. Therefore, in order to find a suitable working system a non-virulent but vaccine strain of *Mycobacterium bovis* BCG was checked for the presence of *whiB* homo-

logues. Genomic DNA of *Mycobacterium bovis* BCG was digested with *Sac*I and *Bam*HI restriction enzymes and the DNA fragments were separated on an 1 per cent agarose gel. The DNA was transferred onto a Hybond Nylon membrane using the published protocol and then the DNA was fixed in the membrane at 80°C for 30minutes. The cloned *whiB* fragments from pUC19 were removed by *Xba*I and *Kpn*I digestion and the DNA was purified using an Gel Extraction kit. The purified fragments were then used for 32p dCTP labeling by random priming kit, which is commercially available. By using standard protocol of Southern hybridization it was confirmed that the four *whiB* genes homologues of *Mycobacterium tuberculosis* H37Rv are present in *Mycobacterium bovis* BCG. The four *whiB* genes of *Mycobacterium bovis* BCG were PCR amplified using oligonucleotide primers of *Mycobacterium tuberculosis* H37Rv. The PCR fragment were cloned and transformed into a *E.coli* strain as described for *Mycobacterium tuberculosis* H37Rv. The DNA sequence of the cloned fragment and sequence alignment using commercial computer software confirmed that the *whiB* gene sequence of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG is identical.

Example 3

[0042] In order to demonstrate the function of *whiB* genes in the life cycle of mycobacteria, the four-*whiB* homologues were disrupted in *Mycobacterium bovis* BCG. By using commercially available computer software Gene-Runner, restriction enzymes sites within the *whiB* sequences were found. Based on the sequence analysis results, following restriction enzyme sites were selected to generate vectors that then can be used for gene disruption in *Mycobacterium bovis* BCG by the process of homologous recombination. To generate a vector, which could be used for gene disruption following constructs were created:

(a) for the *whiB*1 kanamycin cassette was inserted at *Pvu*II site at the position 143;
(b) for *whiB*2 two independent constructs were made at *Mlu*I at the position 138 and at *Hae*III at the position 133;
(c) for *whiB*3 also two constructs were made (1) *Eco*RI site at the position 52, and at the *Cla*I site at the position 85;
(d) for *whiB*4 insertion was at the *Sac*II site at position 192.

[0043] The *whiB* recombinant clones were digested with the restriction enzymes however whenever the recombinant clone had more than one site for a particular enzyme then the purified *whiB* fragments were digested and then ligated with the kanamycin cassette, which codes for kanamycin resistance. The *E. coli* strain was transformed and clones were selected for ampicillin and kanamycin resistance. Since these clones do not have mycobacterial origin of replication they will not survive within the mycobacteria unless they are integrated in the mycobacteria genome. Electrocompetent *Mycobacterium bovis* BCG cells were prepared using the art known in the literature and transformed with 1μg of purified vector DNA. In each tube 1ml of 7H9 Middlebrook's medium was added and incubated at 37°C for 48hrs. The culture was then plated on a 7H10 Middlebrook's medium containing both ampicillin and kanamycin and incubated at 37°C. After three weeks of growth, the colonies were again plated on only kanamycin plates and allowed to grow at 37°C. In the kanamycin plates, the colonies could be seen only after 6 weeks of incubation which suggested that the *whiB* disruption is deleterious for the growth of *Mycobacterium bovis* BCG. Disruption of individual *whiB* genes had same effects on the growth and survival of *Mycobacterium bovis* BCG.

Example 4

[0044] To demonstrate the nature of the *whiB* gene the yeast two-hybrid system was used. This art is well known in the literature. In principle the system has been developed where trans-activation domain and the DNA binding domains are in two different plamids. Unless they come together protein-protein interaction will not take place thus a gene will not get activated. However, the system also allows one to check whether the gene in question codes for DNA binding protein or a transcription activator.

[0045] The three *whiB* genes: *whiB*1, *whiB*2 and *whiB*4 were PCR amplified with a oligonucleotide primers which had *Eco*RI site sequence in the 5' end and *Xho*I site sequence on their 3' end. The *whiB*3 gene was amplified using *Bam*HI site in its 5' end because the *whiB* 3 gene has an internal *Eco*RI site. The PCR conditions were as described earlier. The PCR products were purified using a commercial purification kit and digested either with *Eco*RI and *Xho*I or *Bam*HI and *Xho*I restriction enzymes. The *E. coli* / *Saccharomyces cerevisiae* shuttle vector pEG202 was also digested appropriately, dephospyorylated and the PCR fragments were ligated in to the vector by the method well know in the literature. The recombinants were selected on an ampicillin plate. The ampicillin positive clones were hybridized using the *whiB* genes as probe by the method well established in the literature. Plasmids were recovered from the clones that gave positive signal by using published method. To establish further that correct gene has been cloned the plasmids were digested with appropriate restriction enzymes as described earlier and checked on an agarose gel for the correct size fragment.

[0046] Using a commercially available purification kit, large-scale plasmid preparation of the recombinant clones

was made. Using a published protocol, 1μg of the recombinant plasmid was transformed into *Saccharomyces cerevisiae* EGY48 and EGY191 strains and selected for the complementation of histidine auxotrophy. The clones were patched on a histidine negative plate. The *Saccharomyces cerevisiae* EGY48 and EGY191 strains carrying recombinant *whiB* genes were co-transformed using various combinations of the plasmids:

(a) recombinant pEG202 + pSH18-34 + pJG4-5 (selected for histidine, uracil and tryptophan auxotrophy)
(b) pSH17-4 + pSH18-34+ pJG4-5 (selected for uracil and tryptophan auxotrophy)
(c) pRFHMI + pSH18-34 + pJG4-5 (selected for uracil and tryptophan auxotrophy)
(d) recombinant pEG202 + pJk101 (selected for histidine and uracil auxotrophy)
(e) pRFHM + pJK101 (selected for uracil auxotrophy)
(f) pJK101 (selected for uracil auxotrophy)
(g) recombinant pEG202+ pSH18-34 + pJG4-5 (selected for histidine, uracil and tryptophan auxotrophy).

[0047] Once the transformants complementing for the right auxotrophy were obtained, these clones were sub-cultured on a selective medium lacking those amino acids which was coded by the plasmids. Randomly selected six different clones were then streaked on the medium having following combinations:

(1) glucose $^{+}$, ura^{-} BU salt^{+} +X-gal
(2) galactose/raffinose^{+}, ura^{-} + BU salt^{+} + X-gal
(3) glucose^{+}, ura^{-},his^{-},leu^{-}
(4) galactose/raffinose^{+}, ura^{-}, his^{-}, leu^{-}
(5) galactose/raffinose+, ura-, his-, tryp-, leu-
(6) glucose+, ura-, his-, tryp-, leu-.

The plates containing X- gal were covered with aluminum foil to protect from light and were incubated at 30°C for 18-24hrs.

[0048] The *whiB*1, *whiB*2 and *whiB*3 genes in the combination number (g) did not produce any blue color in an X-gal+, galactose/ raffinose+, ura-, BU+ plates nor it grew on galactose/ raffinose+, ura-, his-, tryp- leu-plates. These combinations also did not induce leucine prototrophy. In contrast the combination (d) repressed the *lacZ* induction when it was grown on an ura-, galactose/ raffinose+, BU+, X-gal+ plates, because they did not produce blue color however the pJk101 alone (combination- f) produced blue color. These results confirmed that the *whiB*1, *whiB*2 and the *whiB*3 genes code for a DNA binding protein but not transcription activator.

[0049] The *whiB*4 gene is a weak transcription activator because unlike the recombinant *whiB*1, *whiB*2, and *whiB*3, the recombinant *whiB*4 in the combination number (g) produced light blue color in an X-gal+, galactose/ raffinose+, ura-, BU+ plates and also showed a weak activation of leucine prototrophy. This example establishes that the mycobacteria regulatory genes can be studied in an eukaryotic system -that is yeast and the *whiB* genes of the *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG either code for DNA binding protein or a transcription activator.

Example 5

[0050] To check whether *whiB* like genes are present in other species of mycobacteria or not, the following species of the mycobacteria were tested for the presence of *whiB* like genes: *Mycobacterium avium. Mycobacterium fortuitum, Mycobacterium gastri, Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium microti, Mycobacterium phlei, Mycobacterium scrofulaceum, Mycobacterium smegmatis* and *Mycobacterium xenopi* using standard and published information. Except in *Mycobacterium fortuitum* which appear to have only *whiB*1 homologue, the *whiB* genes homologues are present in all the species listed. The results suggest that one can use the *whiB* genes of the above species as well to develop a drug target.

Advantages of the present invention:

[0051]

1. The invention provides that the *whiB* genes of the *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG are regulatory genes.
2. The invention described here in also provides that the disruption of *whiB* genes is either lethal or deleterious for the survival of mycobacteria.
3. The invention also provides that the *whiB* genes are used as drug target to search for anti-tuberculosis drugs.

4. The invention described here in provides that the yeast two-hybrid system can be used to study the mycobacterial regulatory genes and then to study to what all genes are controlled by these regulatory genes.
5. This invention is the first report where yeast two-hybrid system has been used to investigate the nature of a protein of *Mycobacterium tuberculosis,* which so far, had only had predicted function.
6. This invention also provides for a method wherein all genetically engineered but truncated regulatory genes can also be studied. These studies are done to delineate the function of different regions of the regulatory genes.
7. The invention also provides means to study protein-protein interactions of mycobacterial genes in a heterologous host that is not infectious thus does not pose a health hazard or demand special laboratory set-up.
8. The invention described in here is a reporter gene based assay that uses a well-characterized gene *lacZ.* The *lacZ* gene codes for the β-galactosidase. The assay of β -galactosidase enzyme is simple thus the method is also HTS compatible.

Claims

1. A reporter gene based method for the screening of anti-tuberculosis drugs comprising using *whiB* like genes (*whiB*1, *whiB*2 whiB3 *whiB*4) present in *Mycobacterium tuberculosis* H37Rv, *Mycobacterium bovis* BCG and *Mycobacterium leprae* having DNA and protein sequence as shown in sequence ID 1 to 4 as drug target for the screening of anti-tuberculosis and anti-leprosis drugs.

2. The presence of functional *whiB*1 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG is essential for their survival.

3. The *whiB*1 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobactrium bovis* BCG code for a DNA binding protein.

4. The *whiB*1 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG can be used for the screening of anti-tuberculosis drugs.

5. The presence of functional *whiB*2 gene is important for the normal growth of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

6. The *whiB*2 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG code for a DNA binding protein.

7. The presence of functional *whiB*3 gene is essential for the normal cell division of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

8. The *whiB*3 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG code for a DNA binding protein.

9. The *whiB*3 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG can be used for the screening of anti-tuberculosis drugs.

10. The *whiB*3 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG can be used for the screening of anti-tuberculosis drugs.

11. The presence of functional *whiB*4 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG is important for their normal growth.

12. The *whiB*4 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobcaterium bovis* BCG code for a DNA binding protein.

13. The drugs against *whiB*2 and the *whiB*4 genes of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG will be particularly useful where drug resistance has developed against the *whiB*1 and *whiB*3 genes or where the *anti-whiB*1 and *anti-whiB3* drugs are allergic or toxic.

14. The source of *whiB* like genes can be *Mycobacterium avium, Mycobacterium fortuitum, Mycobacterium gastri,*

Mycobacterium kansasii, Mycobacterium leprae, Mycobacterium marinum, Mycobacterium microti, Mycobacterium phlei, Mycobacterium scrofulaceum, Mycobacterium smegmatis and *Mycobacterium xenopi* and related organisms.

15. A reporter gene based method for the screening of anti-tuberculosis drugs comprising using *whiB* like genes (*whiB*1, *whiB*2 whiB3 *whiB*4) present in *Mycobacterium tuberculosis* H37Rv, *Mycobacterium bovis* BCG and *Mycobactereium leprae* having DNA and protein sequence as shown in sequence ID 1 to 4 as drug target for the screening of anti-tuberculosis and anti-leprosis drugs, said method comprising the steps of:

(a) amplifying the *whiB* genes of *Mycobacterium tuberculosis* H37Rv,
(b) cloning of the products obtained in step (a) into plasmid vectors such as pUC19 or pBluescript SK^+ or SK^- and transforming into *E.coli;*
(c) amplifying the *whiB* genes of *Mycobacterium bovis* BCG by using the oligonucleotide primers of *Mycobacterium tuberculosis* H37Rv;
(d) cloning the product as obtained in step (c) into a plasmid vector pUC19 or pBluescript SK^+ or SK^-;
(e) constructing a gene disruption integrative vector by inserting a kanamycin cassette in between the *whiB* gene sequences of *Mycobacterium tuberculosis* H37Rv identified as sequence ID1 for *whiB*1, ID 2 for *whiB*2, ID3 for *whiB*3 and ID4 for *whiB*4 ;
(f) constructing a gene disruption integrative vector by inserting a kanamycin cassette in between the *whiB* gene sequences of *Mycobacterium bovis* BCG;
(*g*) amplifying *whiB* genes of *Mycobacterium tuberculosis* H37Rv by using oligonucleotide primers carrying either *Eco*RI, or *Bam*HI restriction enzyme sites at the 5' end and *Xho*I restriction enzyme site at the 3' end;
(h) digesting the fragment as obtained in step (f) with either *Eco*RI or *Bam*HI or *Xho*I restriction enzymes and then cloning of the fragment into a *E. coli*/*Saccharomyces cerevisiae* shuttle vector pEG202 to produce the cloned gene as a *LexA* fusion protein and transforming the recombinant plasmid into a *E.coli;*
(i) preparation of large amount of the desired recombinant plasmid and then transforming the recombinant *whiB*1, *whiB*2, *and whiB*3 / pEG202 into the *Saccharomyces cerevisiae* strains EGY48 and EGY191,
(j) co-transforming pJK101, pSH 18-34, pSH17-4, pJG4-5 and pRFHM vectors into the *Saccharomyces cervisiae* strains EGY48 and EGY191 already harboring *whiB* genes in the pEG202 vector and then selecting for the clones, which complements for uracil and histidine+uracil or histidine+ uracil+tryptophan auxotrophy;
(k) checking the clones for the activation of both *lacZ* and *leu2* reporter genes by inoculating the recombinant clones in to a yeast-nitrogen base medium;
(l) the *whiB*1, *whiB*2 and *whiB*3 genes were co-transformed separately with pJK101 plasmid and selected for histidine and uracil auxotrophy; and
(m) using the DNA binding property of the *whiB*1, *whiB*2 and *whiB*3 to screen for anti-tuberculosis drugs.

16. A method as claimed in claim 15 wherein restriction enzymes are selected from *Eco*RI, *Xma*I *Bam*HI, *Sal*I, *Nco*I, *Not*I, *Xho*I, *Sal*I and *Pst*I.

17. A method as claimed in claim 15 wherein *whiB*1 gene is essential for the survival of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

18. A method as claimed in claim 15 wherein the *whiB*2 gene is essential for the normal growth of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

19. A method as claimed in claim 15 wherein the *whiB*3 gene is essential for the cell division of a *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

20. A method as claimed in claim 15 wherein the *whiB*4 gene is essential for the normal growth of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG.

21. A method as claimed in claim 15 wherein the *whiB*4 gene of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG is a transcription activator.

22. A method as claimed in claim 15 wherein the *whiB*1, *whiB*2 and *whiB*3 genes of *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG code for a DNA binding protein.

23. A method as claimed in claim 15 wherein the whiB1, whiB2 and whiB3 genes of *Mycobacterium tuberculosis* H37Rv

and *Mycobacterium bovis* BCG repress the activation of β- galactosidase genes after binding to the Lex A operator or any other like operators.

24. A method as claimed in claim 15 wherein the DNA binding domain of the *whiB* genes is situated within the 15 amino acids at the carboxy- terminus of the protein.

25. A method as claimed in claim 15 wherein the DNA binding property of the *whiB* genes has been used to screen those anti- tuberculosis drugs which attack the *whiB* genes.

26. A method as claimed in claim 15 wherein the method can be used for any gene, which codes for a DNA binding protein.

27. A method as claimed in claim 15 where the method is compatible to any High — Through- Put or automated screening system.

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 01 30 2991

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	WO 94 28137 A (SATHISH MUNDAYOOR ;SHINNICK THOMAS M (US); KING C HAROLD (US); US) 8 December 1994 (1994-12-08) * page 4, line 15 - page 6, line 25 *	1,15,16, 25,27	C12Q1/68
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1999 MULDER N J ET AL: "Characterization of a Mycobacterium tuberculosis homologue of the Streptomyces coelicolor whiB gene." Database accession no. PREV200000002199 XP002180394 * abstract * & TUBERCLE AND LUNG DISEASE, vol. 79, no. 5, 1999, pages 299-308, ISSN: 0962-8479 -/--	1	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C12Q

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
MUNICH	30 October 2001	Luzzatto, E

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

INCOMPLETE SEARCH SHEET C

Application Number
EP 01 30 2991

Claim(s) searched completely:
15,17-25,27

Claim(s) searched incompletely:
1,16

Claim(s) not searched:
2-14,26

Reason for the limitation of the search (non-patentable invention(s)):

Article 52 (2)(a) EPC - Discovery
Claims 2-14 relate exclusively to features of the whiB genes which are however not put to any practical use and are thus not linked to any patentable subject-matter (entity or process). These claims amount thus to the statement of a discovery (see the Guidelines, C-IV "Discoveries") and are therefore excluded form patentability.

Further limitation of the search

Claim(s) searched incompletely:
1,16

Claim(s) not searched:
26

Reason for the limitation of the search:

1) Claim 1 relates to a "reporter gene based method" solely characterised in that it comprises the use of SEQ IDs 1-4 as drug targets for the screening of anti-tuberculosis or anti-leprosy drugs.
However, the expression "reporter gene based" applies to a great variety of methods, whereas the description only provides some indications as to a method based on the activation of the reporter gene lacZ which produces beta-galactosidase (see p. 3, 2nd full paragraph, p. 16, last 3 lines, claim 15).
With respect to claim 1, therefore, the search has been carried out only insofar as a reporter gene based on the use of the lacZ gene as reporter. It should be emphasised that the fact that a search has been done does not have any bearing on the subsequent assessment as to whether the application and the claims meet the requirements of Art. 83 and 84 EPC, which will be dealt with at the examination stage.
Furthermore, a gene reporter method cannot be based on protein sequences, but requires nucleic acid sequences. The search has been therefore further limited to a method comprising the use of SEQ IDs 1, 3, 5 and 7, in view of the fact that these SEQ IDs 1,3,5 and 7 are the nucleic acid sequences of whiB1-4 genes. The sequence of these genes was designated ID 1-4 in the original sequence listing, which however did not comply with the sequence listing requirements of the EPO.

2) Claim 16, which is dependent on claim 15, relates to a number of restriction endonucleases which go well beyond those listed, in a

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INCOMPLETE SEARCH
SHEET C

Application Number
EP 01 30 2991

limiting way, in claim 15 and whose use is taught in the description (p. 14) (EcoRI, BamHI, XhoI). Claim 16, thus, has only been searched insofar as related to the enzymes listed in claim 15.

3) The description explicitly states that the invention is predicated on the use of the M. tuberculosis whiB genes of SEQ IDs 1-4. Claim 26 is thus fully unsupported (Art. 84 EPC) and has not been searched.

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PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 30 2991

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
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A,D	GOMEZ J.E. ET AL.: "whmD is an essential mycobacterial gene required for proper septation and cell division" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 97, no. 15, 18 July 2001 (2001-07-18), pages 8554-8559, XP002180393 US * the whole document *	1	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	DATABASE SWISS-PROT 'Online! Accession Number: O53353, 1 June 1998 (1998-06-01) COLE S.T. ET AL.: "Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence" XP002180395 * abstract *	1	
	-/--		

EPO FORM 1503 03.82 (P04C10)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 01 30 2991

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	SRIVASTAVA R ET AL: "Beta-galactosidase reporter system in mycobacteria and its application in rapid antimycobacterial drug screening;" BIOCHEM.BIOPHYS.RES.COMMUN.;(1997) 235, 3, 602-05 CODEN: BBRCA9 ISSN: 0006-291X, XP002180396 Cent.Drug-Res.Inst.Lucknow *abstract,discussion* ------	1,15	TECHNICAL FIELDS SEARCHED (Int.Cl.7)

EPO FORM 1503 03.82 (P04C10)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 01 30 2991

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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30-10-2001

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		AU 684798 B2	08-01-1998
		AU 7045294 A	20-12-1994
		CA 2163438 A1	08-12-1994
		DE 69416673 D1	01-04-1999
		DE 69416673 T2	16-09-1999
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		WO 9428137 A1	08-12-1994
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EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82